RayBio®
Human/Mouse/Rat Ghrelin Enzyme Immunoassay Kit

Please Read the Manual Carefully Before Starting your Experiment

User Manual 3.2
(Revised Jan 2, 2014)

RayBio® Ghrelin Enzyme Immunoassay Kit Protocol

(Cat#: EIA-GHR-1)

RayBiotech, Inc.

We Provide You With Excellent Protein Array System and Service

Tel: (Toll Free) 1-888-494-8555 or 770-729-2992; Fax: 770-206-2393;
Web: www.raybiotech.com Email: info@raybiotech.com

RayBiotech, Inc.

RayBio® Human/Mouse/Rat Ghrelin Enzyme Immunoassay Kit Protocol

TABLE OF CONTENTS

I.	Introduction……..	2
II.	General Description……..	3
III.	Reagents……..	5
IV.	Storage……..	5
V.	Additional Materials Required…….	5
VI.	Reagent Preparation…….	6
VII.	Assay Procedure……..…...	9
VIII.	Assay Procedure Summary…….	11
IX.	Calculation of Results…….	11
A.	Typical Data……..	12
B.	Sensitivity…….	13
C.	Detection Range……..	13
D.	Reproducibility…….	13
X.	Specificity…….	13
XI.	References…….	13
XII.	Troubleshooting Guide…….	15

I. INTRODUCTION

Obesity, which is characterized by excessive accumulation of adipose tissue in the body, has become one of the greatest public health challenges. Obesity is not only associated with health problems linked to increased weight-dependent pressure overload on lung, joints and bones, but also a important risk factor for life-threatening diseases such as cardiovascular diseases, type 2 diabetes and certain cancers.

Ghrelin is synthesized as a preprohormone, and then proteolytically processed to yield a 28-amino acid peptide. Synthesis of ghrelin occurs predominantly in epithelial cells lining the fundus of the stomach, with smaller amounts produced in the placenta, kidney, pituitary and hypothalamus.

Ghrelin has emerged as the first circulating hunger hormone. Ghrelin increases food intake and thus fat mass by an action exerted at the level of the hypothalamus. They activate cells in the arcuate nucleus that include the orexigenic neuropeptide Y (NPY) neurons. Ghrelin-responsiveness of these neurons is both leptin and insulin sensitive. Ghrelin also activates the mesolimbic cholinergic-dopaminergic reward link, a circuit that communicates the hedonic and reinforcing aspects of natural rewards, such as food.

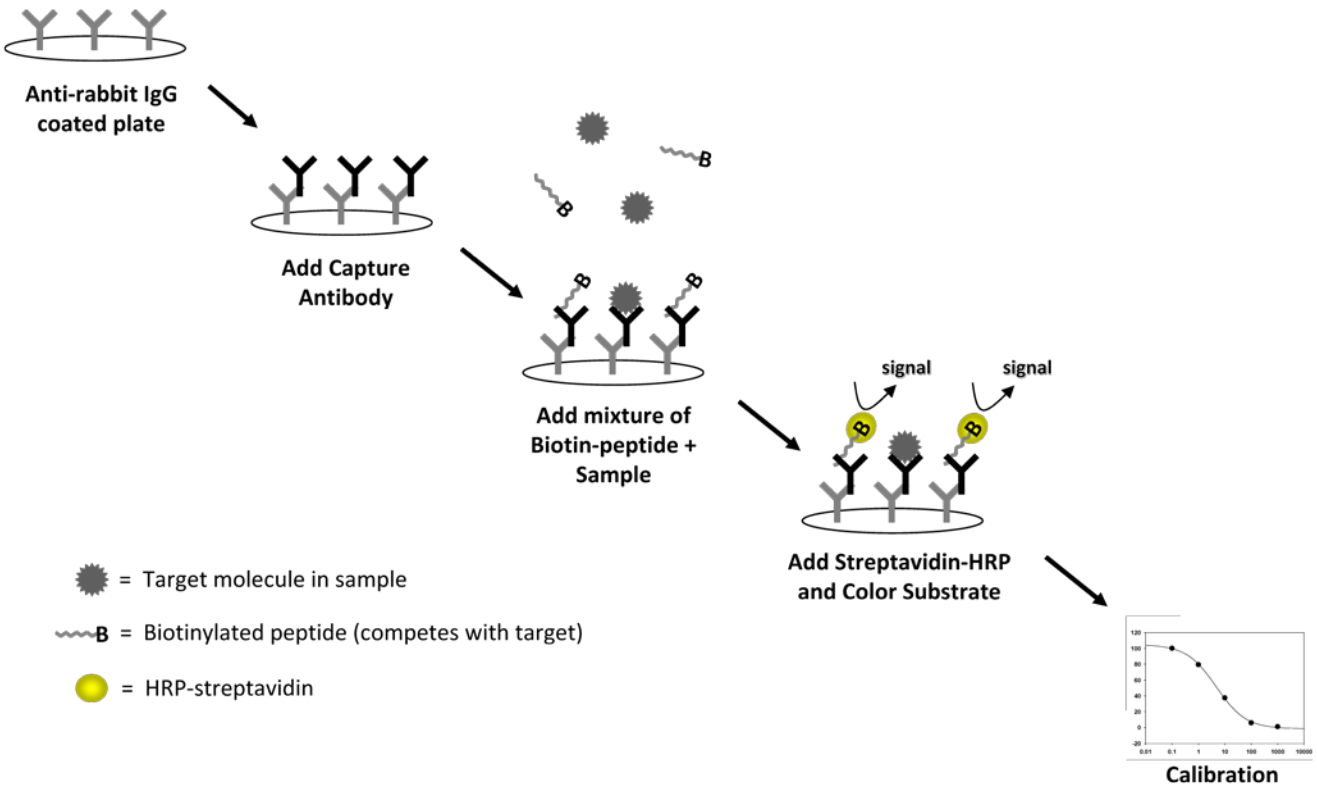
Ghrelin levels in the plasma of obese individuals are lower than those in leaner individuals except in the case of Prader-Willi syndrome-induced obesity. Those suffering from the eating disorder anorexia nervosa have high plasma levels of ghrelin compared to both the constitutionally thin and normal-weight controls. These findings suggest that ghrelin plays a role in both anorexia and obesity. Ghrelin levels are also high in patients who have cancer-induced cachexia.

II. GENERAL DESCRIPTION

The RayBio® Ghrelin Enzyme Immunoassay (EIA) Kit is an in vitro quantitative assay for detecting Ghrelin peptide based on the principle of Competitive Enzyme Immunoassay.

The microplate in the kit is pre-coated with anti-rabbit secondary antibody. After a blocking step and incubation of the plate with anti-Ghrelin antibody, both biotinylated Ghrelin peptide and peptide standard or targeted peptide in samples interacts competitively with the Ghrelin antibody. Uncompeted (bound) biotinylated Ghrelin peptide then interacts with Streptavidin-horseradish peroxidase (SA-HRP), which catalyzes a color development reaction. The intensity of colorimetric signal is directly proportional to the amount of biotinylated peptide-SA-HRP complex and inversely proportional to the amount of Ghrelin peptide in the standard or samples. This is due to the competitive binding to Ghrelin antibody between biotinylated Ghrelin peptide and peptides in standard or samples. A standard curve of known concentration of Ghrelin peptide can be established and the concentration of Ghrelin peptide in the samples can be calculated accordingly.

Principle of Competitive EIA



III. REAGENTS

1. Ghrelin Microplate (Item A): 96 wells (12 strips x 8 wells) coated with secondary antibody.
2. Wash Buffer Concentrate (20x) (Item B): 25 ml.
3. Lyophilized standard Ghrelin peptide (Item C): 2 vials.
4. Lyophilized anti-Ghrelin polyclonal antibody (Item N): 2 vials.
5. 1X Assay Diluent E (Item R): 2 vials, 25 ml/vial. Diluent for both standards and samples including serum or plasma, cell culture media or other sample types.
6. Lyophilized biotinylated Ghrelin peptide (Item F): 2 vials.
7. HRP-Streptavidin concentrate (Item G): 600 µl 100x concentrated HRP-conjugated Streptavidin.
8. Lyophilized positive control (Item M): 1 vial.
9. TMB One-Step Substrate Reagent (Item H): 12 ml of 3, 3', 5, 5'- tetramethylbenzidine (TMB) in buffered solution.
10. Stop Solution (Item I): 8 ml of 0.2 M sulfuric acid.
11. Assay Diagram (Item J).
12. User Manual (Item K).

IV. STORAGE

- Standard, Biotinylated Ghrelin peptide, and Positive Control should be stored at -20°C after arrival. **Avoid multiple freeze-thaws.**
- The remaining kit components may be stored at 4°C.
- Opened Microplate Wells and antibody (Item N) may be stored for up to 1 month at 2° to 8°C. Return unused wells to the pouch containing desiccant pack and reseal along entire edge.
- If stored in this manner, RayBiotech warranties this kit for 6 months from the date of shipment.

V. ADDITIONAL MATERIALS REQUIRED

1. Microplate reader capable of measuring absorbance at 450nm.
2. Precision pipettes to deliver 2 μl to 1 ml volumes.
3. Adjustable 1-25 ml pipettes for reagent preparation.
4. 100 ml and 1 liter graduated cylinders.
5. Absorbent paper.
6. Distilled or deionized water.
7. SigmaPlot software (or other software which can perform four-parameter logistic regression models)
8. Tubes to prepare standard or sample dilutions.
9. Orbital shaker
10. Aluminum foil
11. Saran Wrap

VI. REAGENT PREPARATION

For sample and positive control dilutions, refer to steps 5, 6, 7 and 9 of Reagent Preparation.

1. Keep kit reagents on ice during reagent preparation steps. Equilibrate plate to room temperature before opening the sealed pouch.

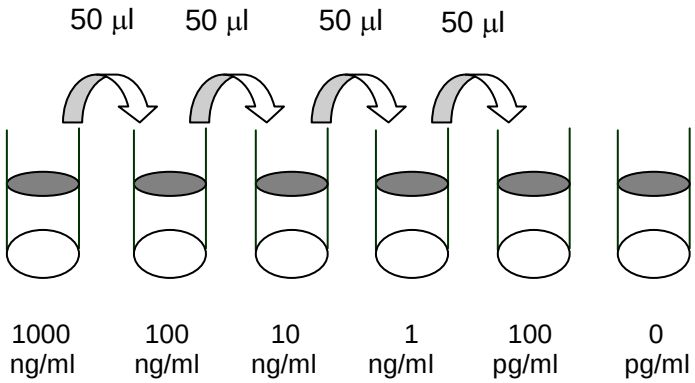
2. Briefly centrifuge the GHR Antibody vial (Item N) and reconstitute with 5 µl of ddH_2O before use. Add 50 µl of 1x Assay Diluent E into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently.

3. The antibody concentrate should then be diluted 100-fold with 1x Assay Diluent E. This is your anti-Ghrelin antibody working solution, which will be used in step 2 of the Assay Procedure.

NOTE: the following steps may be done during the antibody incubation procedure (step 2 of Assay Procedure).

4. Briefly centrifuge the vial of biotinylated Ghrelin peptide (Item F) and reconstitute with 20 µl of ddH_2O before use. Add 5 µl of Item F to 5 ml 1X Assay Diluent E. Pipette up and down to mix gently. *The final concentration of biotinylated Ghrelin will be 10 ng/ml.* This solution will only be used as the diluent in step 5 of Reagent Preparation.

5. Preparation of Standards: Label 6 microtubes with the following concentrations: 1000 ng/ml, 100 ng/ml, 10 ng/ml, 1 ng/ml, 100 pg/ml and 0 pg/ml. Pipette 450 µl of biotinylated Ghrelin solution into each tube, except for the 1000 ng/ml (leave this one empty). *It is very important to make sure the concentration of biotinylated Ghrelin is 10 ng/ml in all standards.*
   a. Briefly centrifuge the vial of standard Ghrelin peptide (Item C) and reconstitute with 10 µl of ddH_2O. In the tube labeled 1000 ng/ml, pipette 8 µl of Item C and 792 µl of 10 ng/ml biotinylated Ghrelin solution (prepared in step 4 above). This is your Ghrelin stock solution (1000 ng/ml Ghrelin, 10 ng/ml biotinylated Ghrelin). Mix thoroughly. This solution serves as the first standard.
   b. To make the 100 ng/ml standard, pipette 50 µl of Ghrelin stock solution the tube labeled 100 ng/ml. Mix thoroughly.
   c. Repeat this step with each successive concentration, preparing a dilution series as shown in the illustration below. Each time, use 450 µl of biotinylated Ghrelin and 50 µl of the prior concentration until 100 pg/ml is reached. Mix each tube thoroughly before the next transfer.
   d. The final tube (0 pg/ml Ghrelin, 10 ng/ml biotinylated Ghrelin) serves as the zero standard (or total binding).



6. Prepare a 10-fold dilution of Item F. To do this, add 2 µl of Item F to 18 µl of 1X Assay Diluent E. This solution will be used in steps 7 and 9.

7. Positive Control Preparation: Briefly centrifuge the positive control vial and reconstitute with 100 µl of ddH_2O before use (Item M). To the tube of Item M, add 101 µl 1x Assay Diluent E. Also add 2 µl of 10-fold diluted Item F (prepared in step 6) to the tube. This is a 2-fold dilution of the positive control. Mix thoroughly. The positive control is a cell culture medium sample with an expected signal between 10% and 30% of total binding (70-90% competition) if diluted as described above. It may be diluted further if desired, but be sure the final concentration of biotinylated Ghrelin is 10 ng/ml.

8. If Item B (20X Wash Concentrate) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of 1X Wash Buffer.

9. Sample Preparation: Use 1X Assay Diluent E + biotinylated GHR to dilute samples, including serum/plasma, cell culture medium and other sample types.

It is very important to make sure the final concentration of the biotinylated Ghrelin is 10 ng/ml in every sample. EXAMPLE: to make a 4-fold dilution of sample, mix together 2.5 µl of 10-fold diluted Item F (prepared in step 6), 185 µl of 1X Assay Diluent E, and 62.5 µl of your sample; mix gently. The total volume is 250 µl, enough for duplicate wells on the microplate.

Do not use Item F diluent from Step 4 for sample preparation. If you plan to use undiluted samples, you must still add biotinylated Ghrelin to a final concentration of 10 ng/ml. EXAMPLE: Add 2.5 µl of 10-fold diluted Item F to 247.5 µl of sample. NOTE: Optimal sample dilution factors should be determined empirically, however you may contact technical support (888-494-8555; techsupport@raybiotech.com) to obtain recommended dilution ranges for serum or plasma.

10. Briefly centrifuge the HRP-Streptavidin vial (Item G) before use. The HRP-Streptavidin concentrate should be diluted 100-fold with 1X Assay Diluent E.

VII. ASSAY PROCEDURE:

1. Keep kit reagents on ice during reagent preparation steps. It is recommended that all standards and samples be run at least in duplicate.

2. Add 100 µl anti-Ghrelin antibody (see Reagent Preparation step 3) to each well. Incubate for 1.5 hours at room temperature with gentle shaking (1-2 cycles/sec). You may also incubate overnight at 4°C.

3. Discard the solution and wash wells 4 times with 1x Wash Buffer (200-300 µl each). Washing may be done with a multichannel pipette or an automated plate washer. Complete removal of liquid at each step is essential to good assay performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add 100 µl of each standard (see Reagent Preparation step 5), positive control (see Reagent Preparation step 7) and sample (see Reagent Preparation step 9) into appropriate wells. Be sure to include a blank well (Assay Diluent only). Cover wells and incubate for 2.5 hours at room temperature with gentle shaking (1-2 cycles/sec) or overnight at 4°C.

5. Discard the solution and wash 4 times as directed in Step 3.

6. Add 100 µl of prepared HRP-Streptavidin solution (see Reagent Preparation step 10) to each well. Incubate with gentle shaking for 45 minutes at room temperature. It is recommended that incubation time should not be shorter or longer than 45 minutes.

7. Discard the solution and wash 4 times as directed in Step 3.
8. Add 100 µl of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking (1-2 cycles/sec).

9. Add 50 µl of Stop Solution (Item I) to each well. Read absorbances at 450 nm immediately.

VIII. ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples and standards as instructed.

⇩

2. Add 100 μl anti-Ghrelin antibody to each well. Incubate 1.5 hours at room temperature or overnight at 4°C.

⇩

3. Add 100 μl standard or sample to each well. Incubate 2.5 hours at room temperature or overnight at 4°C.

⇩

4. Add 100 μl prepared streptavidin solution. Incubate 45 minutes at room temperature.

⇩

5. Add 100 μl TMB One-Step Substrate Reagent to each well. Incubate 30 minutes at room temperature.

⇩

6. Add 50 μl Stop Solution to each well. Read at 450 nm immediately

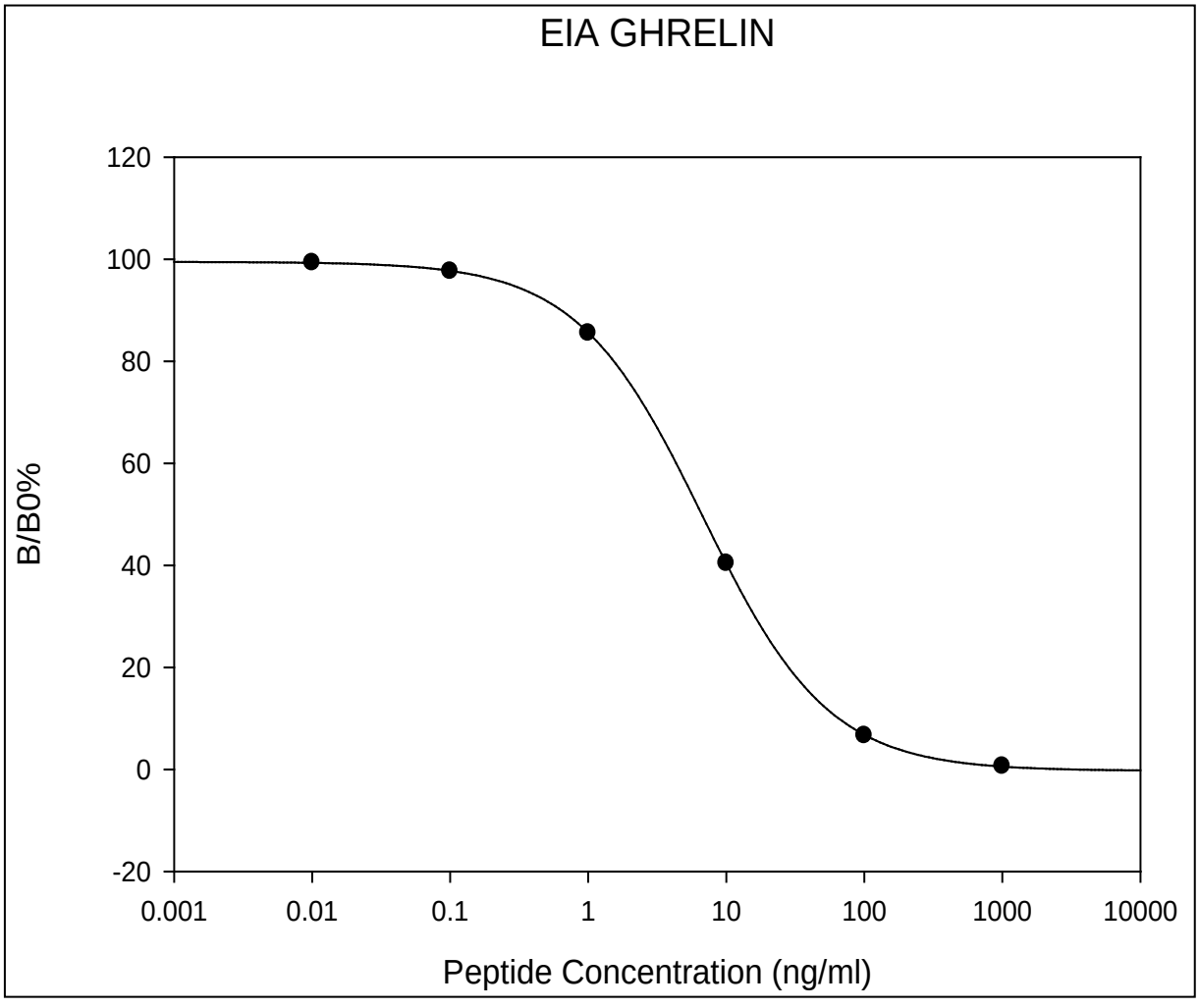
IX. CALCULATION OF RESULTS

Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the blank optical density. Plot the standard curve using SigmaPlot software (or other software which can perform four-parameter logistic regression models), with standard concentration on the x-axis and percentage of absorbance (see calculation below) on the y-axis. Draw the best-fit curve through the standard points.

Percentage absorbance = (B – blank OD)/(B_o – blank OD) where
B = OD of sample or standard and
B_o = OD of zero standard (total binding)

A. TYPICAL DATA

These standard curves are for demonstration only. A standard curve must be run with each assay.



B. SENSITIVITY

The minimum detectable concentration of Ghrelin is 161 pg/ml or 12.46pM.

C. DETECTION RANGE

0.1-1,000 ng/ml

D. REPRODUCIBILITY

Intra-Assay: CV<10%
Inter-Assay: CV<15%

X. SPECIFICITY

Cross Reactivity: This ELISA kit shows no cross-reactivity with any of the cytokines tested: Nesfatin, Angiotensin II, NPY and APC.

XI. REFERENCES

1. Garcia J, Garcia-Touza M, Hijazi R, Taffet G, Epner D, Mann D, Smith R, Cunningham G, Marcelli M (2005). "Active ghrelin levels and active to total ghrelin ratio in cancer-induced cachexia". *J Clin Endocrinol Metab* **90** (5): 2920–6.
2. Yildiz B, Suchard M, Wong M, McCann S, Licinio J (2004). "Alterations in the dynamics of circulating ghrelin, adiponectin, and leptin in human obesity". *Proc Natl Acad Sci U S A* **101** (28): 10434–9.
3. Inui A, Asakawa A, Bowers CY, *et al* (2004). "Ghrelin, appetite, and gastric motility: the emerging role of the stomach as an endocrine organ". *FASEB J.* **18** (3): 439–56.
4. Hewson AK, Tung LY, Connell DW, Tookman L, Dickson SL. (2002). "The rat arcuate nucleus integrates peripheral signals

provided by leptin, insulin, and a ghrelin mimetic". *Diabetes.* **51** (12): 3412–3419.
5. Cummings D, Weigle D, Frayo R, Breen P, Ma M, Dellinger E, Purnell J (2002). "Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery". *N Engl J Med* **346** (21): 1623–30.
6. Tschöp M, Smiley DL, Heiman ML (2000). "Ghrelin induces adiposity in rodents". *Nature* **407** (6806): 908–913.
7. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K (1999). "Ghrelin is a growth-hormone-releasing acylated peptide from stomach". *Nature* **402** (6762): 656–60.

XII. TROUBLESHOOTING GUIDE

Problem	Cause	Solution
1. Poor standard curve	1. Inaccurate pipetting	1. Check pipettes
	2. Improper standard dilution	2. Ensure briefly spin the vial of Item C and dissolve the powder thoroughly by a gentle mix.
2. Low signal	1.Too brief incubation times	1. Ensure sufficient incubation time; assay procedure step 2 change to over night
	2. Inadequate reagent volumes or improper dilution	2. Check pipettes and ensure correct preparation
3. Large CV	1. Inaccurate pipetting	1. Check pipettes
4. High background	1. Plate is insufficiently washed	1. Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed.
	2. Contaminated wash buffer	2. Make fresh wash buffer
5. Low sensitivity	1. Improper storage of the EIA kit	1. Store your standard at ≤ -20^{o}C after receipt of the kit.
	2. Stop solution	2. Stop solution should be added to each well before measure

RayBio® EIA kits:

If you are interested in other EIA kits, please visit www.raybiotech.com for details.

Notes:

This product is for research use only.

©2013 RayBiotech, Inc.

3607 Parkway Lane, Suite 200
Norcross, GA 30092
Tel: 770-729-2992, 1-888-494-8555
Fax: 770-206-2393
Web: www.raybiotech.com